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Research Report

Nimodipine accelerates the postnatal development of parvalbumin and S-100 β immunoreactivity in the rat brain

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Abstract

The effects of chronic maternal perinatal nimodipine treatment on the immunocytochemical distribution of the Ca²⁺-binding proteins parvalbumin (PV) and S-100 β in neocortex and hippocampus were studied at the age of postnatal day (PD) 5, 7, 10, 14 and 20. The Ca²⁺ antagonist nimodipine (1000 ppm BAY e 9736 in daily food) was administered to pregnant rats starting at postconceptual day 11. The expression of PV exemplified in layer V of parietal and retrosplenial cortex and in all hippocampal layers of CA1 and CA3 was enhanced by more than two-fold in the offspring of nimodipine-treated dams at PD 10 compared with placebo-treated animals. The difference was no longer present at PD 14 and 20. Nimodipine administration also significantly increased the number of S-100 β -immunopositive glial cells in upper neocortical layers I–III at PD 5 and 7. Again, the difference between nimodipine and placebo-treated animals disappeared after PD 10. The data indicate an accelerated development of PV and S-100 immunoreactivity in the postnatal forebrain as a result of perinatal blockade of the L-type Ca²⁺ current.

Key words: Calcium-binding protein; Parvalbumin; S-100 β ; Immunocytochemistry; Hippocampus; Neocortex

1. Introduction

Ca²⁺ ions play a pivotal role in a large number of processes in the brain. The intracellular free Ca²⁺ concentration [Ca²⁺]_i has been proposed to control events, such as transmitter release, membrane permeability and activation of several enzymes and genes [10,21]. Processes accompanying cellular plasticity are also regulated by mechanisms linked to the [Ca²⁺]_i [11,14]. The period of neuronal development involves dynamic alterations in the structure and position of cells in the CNS during cell migration, neurite outgrowth and synaptogenesis. Several findings indicate a narrow range of the [Ca²⁺]_i for optimal neurite outgrowth and growth-cone motility [14,15,30,37]. Maintenance of intracellular Ca²⁺ homeostasis during development is, therefore, of major importance. This homeostasis is maintained by the concerted action of Ca²⁺-importing and -exporting systems [4,5,16,22]. Cy-

tosolic free Ca²⁺ ions in both neuronal and glial cells can also bind in a fast but transient way to neuronal or glial intracellular Ca²⁺-binding proteins (CaBPs) [1,3], like calbindin-D28k, parvalbumin (PV) and S-100 β . Although little is known yet about the functions of CaBPs, these CaBPs belong to a group often defined as “buffer” proteins because of their presumed Ca²⁺-buffering capacities [3,13,17].

The expression of a neuronal and a glial representative of CaBPs, PV and S-100 β , respectively, is subject of this study. PV is expressed in a subpopulation of fast-firing cells some of which are GABA interneurons. By influencing the activity of Ca²⁺-dependent K⁺ channels, it is thought to regulate the firing frequency of neurons [6]. In this respect, the ontogenic appearance of the protein has been reported to be in temporal association with the beginning of physiological activity of certain inhibitory neurons [35]. PV is also suggested to protect neurons against excessive [Ca²⁺]_i in pathological conditions, like ischemia or seizures [23,34]. The other CaBP that is subject of this study is the glial S-100 β . S-100 is involved in the regulation of

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several enzymatic activities [20,27] and interacts with cytoskeletal functions [8,27,31,33]. Because glial cells are able to secrete S-100 [33], these effects are present not only in astroglial cells [32] but also in neurons. In this way, S-100 is thought to stimulate growth of serotonergic neurons [2,40]. The above-mentioned effects of S-100 on growth and proliferation of cells in the nervous system led to the suggestion that the protein may help coordinate neuronal development.

We recently reported that pre- and early-postnatal hypoxia transiently retarded the ingrowth of serotonergic and cholinergic fibers in neocortex and hippocampus [26]. The effects of such perinatal hypoxic events were also reflected in the impaired adult cognitive and motivational behaviors in which the above-mentioned neurotransmitter systems play a prominent role [24–26]. Cytosolic neuronal Ca^{2+} overload in vulnerable brain regions, like cortex and hippocampus elicited by the perinatal hypoxia [7,38], may well be responsible for the observed anatomical and behavioral effects. This hypothesis finds support in the finding that chronic perinatal administration of the L-type Ca^{2+} channel antagonist nimodipine during hypoxia prevented both suppression of cholinergic and serotonergic innervation of cortex and hippocampus [26] as well as behavioral abnormalities [24,25]. The neuroprotective action of the Ca^{2+} antagonist during perinatal hypoxia may be caused by a decreased Ca^{2+} influx. Another option may be the influence of nimodipine on the Ca^{2+} -buffering capacity of developing neuronal and glial cells. The studies on the effects of nimodipine during development so far have been restricted to its neuroprotective character in combination with hypoxic events while the action of nimodipine in normal brain development remains unclear. Therefore, we investigated the early-postnatal effect of chronic perinatal nimodipine treatment on the distribution of the neuronal CaBP PV and glial S-100 β in neocortex and hippocampus. The effect of nimodipine on the expression of calbindin D-28k, being another neuronal CaBP, was presented in another manuscript (P.G.M. Luiten, submitted). The study of these proteins may yield further insight in neuronal and glial mechanisms of intracellular Ca^{2+} balance during development.

2. Materials and methods

2.1. Animals

The dams were housed in a temperature- and light-controlled room ($21 \pm 1^\circ\text{C}$; 12/12 h light/dark cycle, lights on at 07:00). The day of birth was taken as postnatal day (PD) 1. Within the first 2 days after delivery, the litters were reduced to eight per nest. In total, 57 male pups were sacrificed at PD 5, 7, 10, 14 and 20 from the two groups of animals. To an age group, only one single pup per nest was used to avoid the effect of litter.

2.2. Treatment

Pregnant rats were treated with nimodipine (1000 ppm BAY e 9736 in daily food), starting at postconceptual day 11. Control animals received placebo food pellets. After delivery, the treatment was continued throughout the experiment. The selection of the single dose of the Ca^{2+} antagonist was based on the results of previous immunocytochemical and behavioral studies performed in this laboratory [24–26].

2.3. Immunocytochemical procedure

The rat pups were transcardially perfused under deep pentobarbital anesthesia with cold heparinized saline. After blood removal, the prerinse was followed by a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH = 7.4). The brains were removed from the skull, postfixed for 6 h in the same fixative and cryoprotected overnight at 4°C in 30% buffered sucrose. Coronal sections of 20 μm were cut on a cryostat microtome and collected in phosphate-buffered saline (PBS) (0.01 M, pH = 7.4). The tissue sections were preincubated for 15 min in 0.01% H_2O_2 in PBS, subsequently rinsed in PBS and immersed for 30 min in 5% normal sheep (for PV-staining) or normal goat (in case of S-100-staining) serum to reduce aspecific binding in the following step. Next, the sections were incubated overnight at 4°C with the first antibody (respectively, monoclonal mouse anti-PV IgG (Sigma); 1:2000 in PBS and polyclonal rabbit anti-S-100 β (Sigma); 1:10,000 in PBS). After rinsing, sections were incubated again in appropriate normal sera for 30 min and subsequently exposed to biotinylated sheep anti-mouse IgG (Zymed, 1:200 in PBS, 2 h at room temperature) or goat anti-rabbit IgG (Zymed, 1:50 in PBS, 2 h at room temperature). This step was followed by incubation in streptavidin-HRP (Zymed, 1:200 in PBS, 2 h at room temperature) or rabbit PAP (Zymed, 1:500 in PBS, 2 h at room temperature). Finally, the tissue-bound peroxidase was visualized by reaction with diaminobenzidine (30 mg/100 ml Tris-HCl) and 0.01% H_2O_2 . Sections of placebo- and nimodipine-treated animals were pooled and simultaneously processed for the PV and S-100 β immunostainings. Standard control experiments were performed by omission of the primary antibody, always yielding absence of detectable immunolabeling.

2.4. Quantitative analysis

Quantitative analysis was carried out by visual cell counts of selected hippocampal and cortical areas. Two coronal sections were selected at the level of the dorsal hippocampus corresponding to the anterior–posterior coordinate -3.2 to -3.8 mm relative to Bregma in the adult rat according to the brain atlas of Paxinos and Watson [28]. Hippocampal and cortical cell counts were performed bilaterally and the mean values of four counts per animal were processed for subsequent statistical analysis.

All visible immunoreactive PV neurons were counted within the layers of the parietal cortex defined by Zilles [41] as the Par1 area and in the retrosplenial granular cortex (RSG). No distinction was made between light and strong immunoreactivity. In the dorsal hippocampus, the cell number was added up over the three layers of CA1 and CA3, respectively.

The development of S-100 β in glial cells was also studied at the brain level mentioned above. Since the density of S-100 β -positive glial cells was relatively high, cells were counted within a specified area of an ocular square grid (unit area: $7.9 \mu\text{m} \times 79 \mu\text{m}$ at a $\times 200$ magnification). S-100-positive cells were quantified in Par1 and in hippocampal layers of CA1 and CA3.

The number of animals in the age groups of 5-, 7-, 10-, 14- and 20-day-old rats varied from three to eight.

2.5. Statistics

The results are presented as mean \pm S.E.M. One-way ANOVA was applied for statistical evaluation of treatment effects within age groups. A *P* level of <0.05 was taken as statistical significance for the tests.

3. Results

3.1. Normal distribution of PV-positive interneurons

At PD 5, PV-immunolabeled interneurons could not be detected in hippocampus or neocortex. From PD 7,

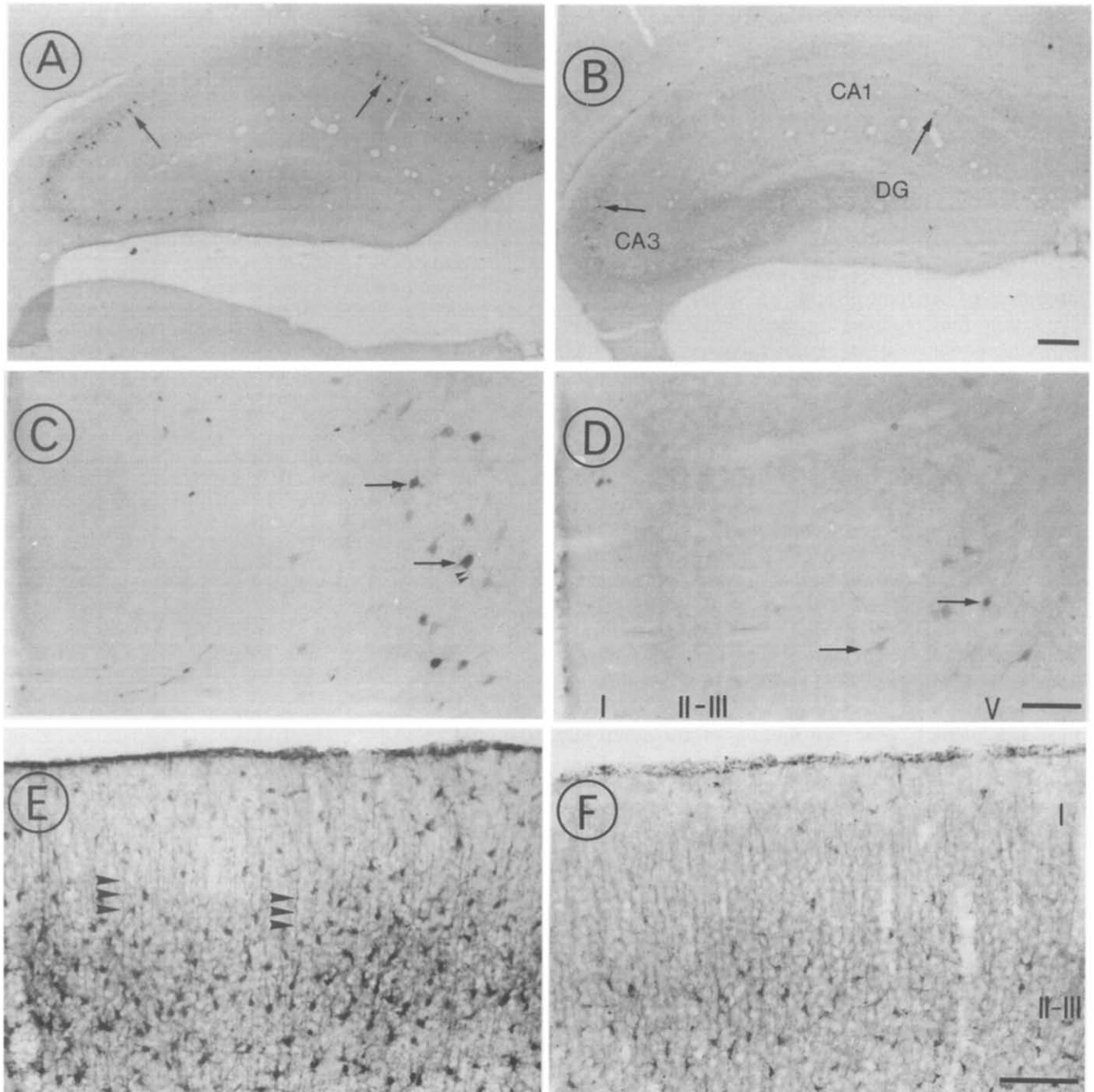


Fig. 1. Photomicrographs of PV (A–D) and S-100 β (E,F) in hippocampus and neocortex of nimodipine- (left panel) and placebo-treated animals (right panel). A,B: low-power photomicrograph showing PV-positive cells in hippocampus of nimodipine- (A) and placebo-treated (B) animal at PD 10, demonstrating higher number of PV-positive interneurons (arrows) in CA1–CA4 of nimodipine-treated rat. C,D: cell bodies (arrows) and processes (arrowheads) labeled in retrosplenial granular cortex (PD 10). Less cells and processes are stained in placebo-treated animals. E,F: S-100-positive astrocytes in layers I–III in parietal cortex (PD 7). Radial glial fibers (arrowheads) are much more distinct in nimodipine-treated rats. Bars in A,B 100 μ m; in C,D 50 μ m and in E,F 50 μ m.

cells appeared in the dorsal hippocampal subicular area and CA3. In the neocortex, PV interneurons started to appear in the RSG and some sparse cells in the parietal cortex. In this early phase, the staining of the cells was still very light and processes were only faintly visible. At PD 10, the number and staining intensity of labeled cells increased in the hippocampal subiculum and CA3 and CA4. Interneurons also became visible in hippocampal CA1. In Par1 and RSG, the majority of PV neurons were situated in pyramidal cell layer V. At PD 14, interneurons started to appear also in cortical layers II, III and VI and, at PD 20, positively labeled cells were seen in all layers. Staining intensity and length of cell processes increased gradually with increasing age.

3.2. Normal distribution of S-100 β -positive glial cells

S-100 β -positive glial cells were already present in large amounts in neocortical brain areas as well as in hippocampus at PD 5. Many of the S-100-positive cells in the neocortex could be classified as radial glial cells because of the high amount of extending immunopositive radial fibers (Fig. 1E,F). In Par1, labeled cells appeared in the classical inside-out organization; many cells present in the deeper cortical layers IV–VI but very little in the upper layers I–III. In all hippocampal areas, many positively stained glial cells were observed

with the highest density in subiculum and CA3 and less in CA1 and dentate gyrus. From PD 7 to 10, the density of labeled cells decreased slightly in hippocampal CA3 and increased in the other hippocampal areas. In Par1, the number of cells remained stable in layers IV–VI and increased in upper layers I–III. From PD 10, the adult distribution pattern in the selected brain areas was reached since the number of S-100-positive cells did not show any further increase.

3.3. Effect of perinatal nimodipine treatment on development of PV- and S-100 β -positive cells

Number of PV-positive cells

Perinatal nimodipine treatment increased the number and enhanced staining intensity of PV-positive interneurons in all neocortical and hippocampal regions studied at PD 10 (Fig. 2). The accumulated cell number in stratum oriens, pyramidale and radiatum of CA1 ($F_{1,12} = 6.0$; $P = 0.03$) and CA3 ($F_{1,12} = 6.2$; $P = 0.03$) was significantly higher in nimodipine-treated animals (Fig. 1A,B). In layer V of Par1 and RSG (Fig. 1C,D), the difference between placebo- and nimodipine-treated groups was even stronger ($F_{1,12} = 9.5$; $P = 0.009$) and ($F_{1,12} = 21.3$; $P = 0.001$, respectively). At PD 14, PV-positive interneurons also started to appear in the more superficial layers of Par1 and RSG. However, in none of the regions, the difference between

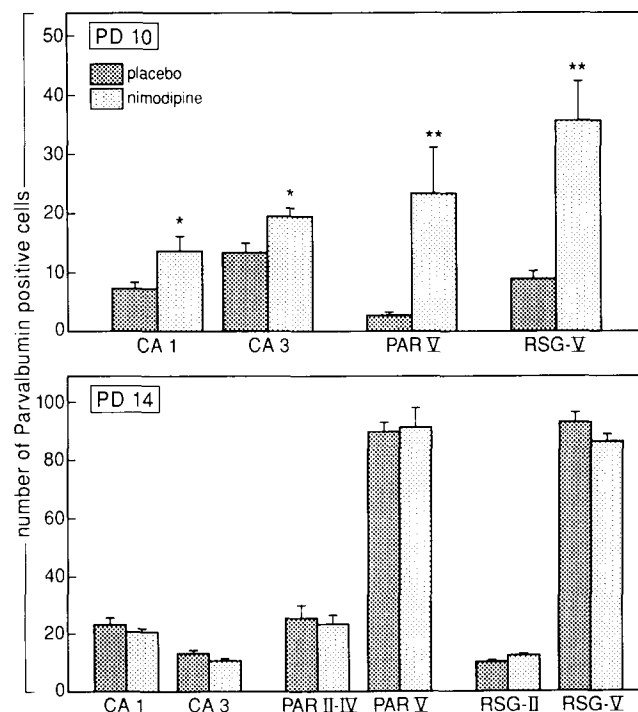


Fig. 2. Effects of nimodipine administration on number of PV-positive cells in hippocampal CA1 and CA3, in layer V of parietal cortex (PAR) and in RSG at PD 10 and 14. Mean \pm S.E.M. values are shown. * $P < 0.05$; ** $P < 0.01$, determined by one-way ANOVA. Number of animals used per group: PD 10: placebo ($n = 8$); nimodipine ($n = 6$). PD 14: placebo ($n = 5$); nimodipine ($n = 6$).

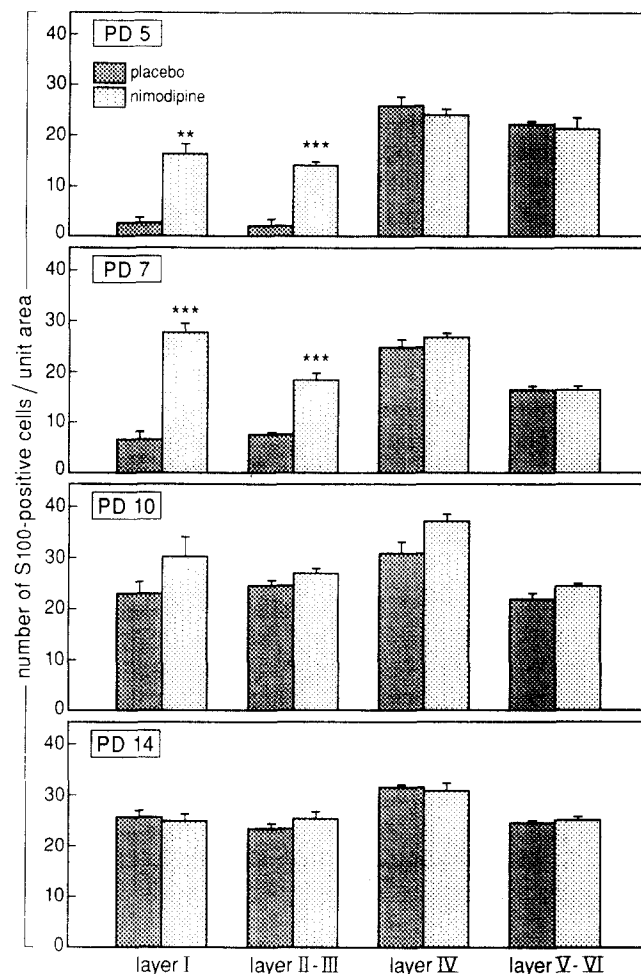


Fig. 3. Effects of nimodipine administration on number of S-100 β -positive astrocytes per unit area in layers I–VI of parietal cortex at PD 5, 7, 10 and 14. Mean \pm S.E.M. values are shown. ** $P < 0.01$; *** $P < 0.001$, determined by one-way ANOVA. Number of rats used per group: PD 5: plac ($n = 8$); nimo ($n = 3$). PD 7: plac ($n = 4$); nimo ($n = 7$). PD 10: plac ($n = 6$); nimo ($n = 5$). PD 14: plac ($n = 6$); nimo ($n = 6$).

any treated and control cases was significant. At PD 20 (data not shown), also no differences between the groups were present.

Number of S-100 β -positive cells

No differences could be revealed between placebo- and nimodipine-treated animals in number of immunopositive glial cells in hippocampal regions. For that reason, no results of cell counts in hippocampus are shown. In Fig. 3, the number of S-100 β cells per unit area ($79 \times 7.9 \mu\text{m}$) in the cortical regions is presented at PD 5, 7, 10 and 14. At PD 5, significantly more S-100 cells were counted in layer I ($F_{1,9} = 28.5$; $P = 0.001$) and layers II and III ($F_{1,9} = 37.7$; $P = 0.000$) of nimodipine-treated rats compared with placebo-treated animals (Fig. 1E,F). In the deeper layers IV–VI, no significant differences were present at PD 5. At PD 7, the differences seen at PD 5 still existed both in layer I ($F_{1,8} = 56.7$; $P = 0.000$) and layers II and III ($F_{1,8} = 41.0$; $P = 0.000$). At PD 10 and 14, the nimodipine- and placebo-treated cases showed similar numbers

of immunoreactive glia. Comparison of ages showed that adult patterns in the deeper cortical layers IV–VI were reached already at PD 5 whereas the more superficial layers I–III yielded an adult distribution at PD 10.

4. Discussion

The present study shows the effects of chronic perinatal administration of the Ca^{2+} antagonist nimodipine on the development of PV and S-100 β immunoreactivity in the rat neocortex and hippocampus. The data indicate that the development of PV and S-100 immunoreactivity in defined postnatal brain regions is accelerated in nimodipine-treated animals. The observed developmental pattern of PV immunoreactivity in neurons and S-100 β in glial cells in placebo-treated animals is in agreement with previous reports [9,35,36].

The effect of nimodipine appears to be transient as it could be observed only in the initial phase of expres-

sion of both PV and S-100. The normal onset of S-100 β immunoreactivity in glial cells precedes that of PV in neuronal cells. This probably causes nimodipine to affect the appearance of S-100 at PD 5 and 7 whereas the effect on PV expression was present first and only at PD 10. It is not clear why nimodipine affects the onset of the CaBPs in specific regions. If it would be a general effect on the onset of expression of the described proteins, one would also expect accelerated development of PV in upper layers of the cortex. Since this is not the case, it appears that nimodipine exerts a cell-type and region-specific action, possibly based on a differential temporal expression of Ca²⁺ channels during development.

By antagonizing the Ca²⁺ influx through voltage-dependent L-type Ca²⁺ channels [19], nimodipine is suggested to offer protection against a deranged Ca²⁺ balance during anoxic or ischemic insults [38]. In view of the hypothesized Ca²⁺-buffering capacities of CaBPs, the accelerated expression of PV and S-100 by perinatal nimodipine might offer another route of cellular protection against a damaging Ca²⁺ influx. Perinatal nimodipine administration not only accelerates the ontogeny of PV and S-100 proteins but also of calbindin D-28k (P.G.M. Luiten et al., submitted). The protective effect of CaBPs against a high [Ca²⁺]_i, however, is still in question. In a number of studies, CaBPs are suggested to offer cellular protection against abnormal increases in [Ca²⁺]_i during ischemia and anoxia [17,23,29,34,39]. In other studies, however, no consistent association between the expression of CaBPs and the predisposition of neurons to ischemic cell death was found [1,12].

Nimodipine application around birth not only accelerates the onset of CaBPs. Chronic perinatal nimodipine administration combined with mild postnatal asphyxia caused a transient increase in serotonergic and cholinergic fiber ingrowth in hippocampus and cortex between PD 5 and 10 [26]. These findings, together with the current results, suggest that perinatal application of nimodipine accelerates several parameters in various transmitter systems during the process of maturation. The nimodipine effects on growth processes in the brain can not be accounted for simply in terms of general acceleration or deceleration of development. Brain and body weights of control- and nimodipine-treated animals within the age groups used in the present study were similar.

The effect of nimodipine can be elicited via a number of mechanisms. Given the importance of the [Ca²⁺]_i in neuronal growth-cone behavior [14,15], it is tempting to propose an effect of nimodipine on growth acceleration by blockade of the L-type Ca²⁺ current [19]. This suggestion is supported by the finding that also certain growth factors act by reducing Ca²⁺ levels in the cell [18]. Nimodipine administration may optimize growth

conditions by holding the [Ca²⁺]_i on the so-called "calcium set-point" [15] by CaBPs.

The present results show an effect of perinatal nimodipine administration on the developmental time of onset of neuronal and glial CaBPs. Retardation of ingrowth of cholinergic and serotonergic fibers after perinatal hypoxic events was also of a transient nature [26]. The behavioral performance, which we associated with the hypoxic insult, however, was permanently disturbed [24,25]. This indicates that, although placebo-treated animals apparently catch up with the delay in neuronal development of certain brain systems, the organization of the brain may remain structurally different after perinatal nimodipine treatment.

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